Figure 5 illustrates an investigational device (by Medtronic, Inc. of Minneapolis, MN - schematic of Model 8506), which can be implanted subcutaneously on the cranium, and provides an access port through which therapeutic agents may be delivered to the brain.

Figure 6 illustrates the relation of various neurodegenerative diseases described herein, and the location of treatment with small interfering RNA vectors directed to their intended targeted gene product.

Figure 7 is a schematic side view depiction of a marker tip at the distal end of the catheter used in implementing the method of the invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention solves two problems in the prior art at the same time: (1) the problem of how to treat neurodegenerative diseases caused by the production in neurons of a protein that has pathogenic properties and (2) the problem of delivery of therapeutic small interfering RNA to affected neurons.

In order to better understand the present invention, a list of terms and the scope of understanding of those terms is provided below.

Terminology

By "alpha-synuclein, BACE1 (including variants thereof, e.g. variants A, B, C, and D), huntingtin, ataxin-1, ataxin-3, and/or atrophin-1 proteins" is meant, a protein or a mutant protein derivative thereof, comprising the amino-acid sequence expressed and/or encoded by alpha-synuclein (Parkinson's disease), and beta-site APP-cleaving enzyme (BACE1 (including variants thereof, e.g. variants A, B, C, and D)) (Alzheimer's disease), huntingtin (Huntington's disease), and ataxin-1 (Spinocerebellar Ataxia Type 1), ataxin-3 (Spinocerebellar Ataxia Type 3 or Machado-Joseph's Disease), and/or dentatorubral-pallidoluysian atrophy (DRPLA) genes and/or the human genomic DNA respectively.

As used herein "cell" is used in its usual biological sense, and does not refer to an entire multicellular organism. The cell may be present in an organism which may be a human but is preferably of mammalian origin, e.g., such as humans, cows, sheep, apes, monkeys, swine, dogs, cats, and the like. However, several steps of producing small

By "small interfering RNA" is meant a nucleic acid molecule which has comp ementarity in a substrate binding region to a specified gene target, and which acts to specifically guide enzymes in the host cell to cleave the target RNA. That is, the small interfering RNA by virtue of the specificity of its sequence and its homology to the RNA target, is able to cause cleavage of the RNA strand and thereby inactivate a target RNA molecule because it is no longer able to be transcribed. These complementary regions allow sufficient hybridization of the small interfering RNA to the target RNA and thus permit cleavage. One hundred percent complementarity often necessary for biological activity and therefore is preferred, but complementarity as low as 90% may also be useful in this invention. The specific small interfering RNA described in the present application are not meant to be limiting and those skilled in the art will recognize that all that is important in a small interfering RNA of this invention is that it have a specific substrate binding site which is complementary to one or more of the target nucleic acid regions.

Small interfering RNAs are double stranded RNA agents that have comp ementary to (i.e., able to base-pair with) a portion of the target RNA (generally messenger RNA). Generally, such complementarity is 100%, but can be less if desired, such as 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%. For example, 19 bases out of 21 bases may be base-paired. In some instances, where selection between various allelic variants is desired, 100% complementary to the target gene is required in order to effectively discern the target sequence from the other allelic sequence. When selecting between allelic targets, choice of length is also an important factor because it is the other factor involved in the percent complementary and the ability to differentiate between allelic differences.

The small interfering RNA sequence needs to be of sufficient length to bring the small interfering RNA and target RNA together through complementary base-pairing interactions. The small interfering RNA of the invention may be of varying lengths. The length of the small interfering RNA is preferably greater than or equal to ten nucleotides and of sufficient length to stably interact with the target RNA; specifically 15-30 nucleotides; more specifically any integer between 15 and 30 nucleotides, such as 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, and 30. By "sufficient length" is meant

these and other devices and systems may be suitable for delivery of small interfering RNA vectors for the treatment of neurodegenerative diseases in accordance with the present invention.

In one preferred embodiment, the method further comprises the steps of implanting a pump outside the brain, the pump coupled to a proximal end of the catheter, and operating the pump to deliver the predetermined dosage of the at least one small interfering RNA or small interfering RNA vector through the discharge portion of the catheter. A further embodiment comprises the further step of periodically refreshing a supply of the at least one small interfering RNA or small interfering RNA vector to the pump outside said brain.

Thus, the present invention includes the delivery of small interfering RNA vectors using an implantable pump and catheter, like that taught in U.S. Patent No. 5,735,814 and 6,042,579, and further using a sensor as part of the infusion system to regulate the amount of small interfering RNA vectors delivered to the brain, like that taught in U.S. Patent No. 5,814,014. Other devices and systems can be used in accordance with the method of the present invention, for example, the devices and systems disclosed in U.S. Serial Nos. 09/872,698 (filed June 1, 2001) and 09/864,646 (filed May 23, 2001), which are incorporated herein by reference.

It is preferred to place some means for locating distal end 14 during the access and location process. This is preferably done by applying a marker 46, as shown in FIG. 7, to distal end 14 which is detected during the access and location process. If access and location is accomplished using some form of x-ray radiation, marker 46 is preferably radiopaque. Radiopaque marker 46 renders at least a portion of distal tip 14 opaque to x-rays, enabling the tip 34 to be observed via fluoroscopy or via x-ray during access and location of catheter 10.

In a preferred embodiment, radiopaque marker 46 comprises tantalum powder dispersed in a matrix composed of a biocompatible adhesive, such as those discussed above. Ordinarily, radiopaque marker 46 will be premolded prior to insertion into the lumen 38. After radiopaque marker 46 has been inserted into the lumen 38, a thin coating of the same biocompatible adhesive is preferably applied to the exterior of the hemispherical portion 48. Other materials may also be suitable for radiopaque marker 46,

such as barium or platinum materials.

Alternately, the radiographic marker 46 may be chosen of a material that has sufficient radiodensity for visualization during radiologic procedures, but in powdered form that is dispersed in the catheter tip 34 at the time the catheter tip 34 is molded.

Alternatively, marker 46 may be composed of a material that is compatible to nuclear magnetic resonance imaging (MRI) to enable the tip 34 to be detected during an MRI scan. Preferred material for such a marker 46 is platinum, though barium, tantalum, and similar materials are also suitable. Regardless of whether radiography or MRI is being utilized, the goal of providing a radiographic marker 46 is to enable the operator to accurately detect the precise location of the tip 34 to facilitate placement and later verification of the integrity and position of distal end 14 of catheter 10.

To summarize, the present invention provides methods to deliver small interfering RNA vectors to the human central nervous system, and thus treat neurodegenerative diseases by reducing the production of a pathogenic protein within neurons.

The present invention is directed for use as a treatment for neurodegenerative disorders and/or diseases, comprising Alzheimer's disease, Parkinson's disease, Huntington's disease, Spinocerebellar type 1, type 2, and type 3, and/or any neurodegenerative disease caused or aggravated by the production of a pathogenic protein, or any other neurodegenerative disease caused by the gain of a new, pathogenic function by a mutant protein.

EXAMPLES

Example 1: Construction of a small interfering RNA targeting human ataxin1 mRNA.

As an example of the embodiments of the invention, we have made a small interfering RNA that targets the mRNA for human ataxin1. This small interfering RNA reduces the amount of mRNA for human ataxin1 in human cells, in cell cultures. As a therapy for Spinocerebellar Ataxia Type 1 (SCA1), this same small interfering RNA or a similar small interfering RNA will be delivered to the cells of the cerebellum in the patient's brain, using implanted access ports and catheters. The result will be a reduction

in the amount of ataxin1 protein in these cells, thereby slowing or arresting the progression of the patient's SCA1 disease.

The small interfering RNA against human ataxin1 was been constructed from the nucleotide sequence for human ataxin1. The sequence from human ataxin 1 was retrieved from the publicly-accessible nucleotide database provided by NCBI, retrievable as NCBI accession number NM_000332 (SEQ ID:15). A portion of the human mRNA sequence for ataxin1 was identified as a potential site for small interfering RNA cleavage and also predicted to be single-stranded by MFOLD analysis. In accession NM_000332 (SEQ ID:15), three pairs of anti ataxin1 siRNA targets were constructed:

1. Anti-ataxin1 siRNA targeting the mRNA sequence at sites numbered 945 through 965:

SEQ ID:1 5' - AACCAAGAGCGGAGCAACGAA - 3'
SEQ ID:2 3' - GGTTCTCGCCTCGTTGCTTAA - 5'

2. Anti-ataxin1 siRNA targeting the mRNA sequence at sites numbered 1671 - through 1691:

SEQ ID:3 5' - AACCAAGAGCGGAGCAACGAA - 3'
SEQ ID:4 3' - GGTTCTCGCCTCGTTGCTTAA - 5'

3. Anti-ataxin1 siRNA targeting the mRNA sequence at sites numbered 2750 - through 2770:

SEQ ID:4 5' - AACCAGTACGTCCACATTTCC - 3'
SEQ ID:6 3' - GGTCATGCAGGTGTAAAGGAA - 5'

A series of six deoxyoligonucleotide fragments were designed, ordered and purchased from the MWG Biotech, Inc., custom oligonucleotide synthesis service to provide the six fragments making up the three target sites. Additionally, these oligonucletides were constructed to include an 8 base sequence complementary to the 5' end of the T7 promoter primer included in an siRNA construction kit (Ambion, Inc. catalog number 1620). Each specific oligonucleotide was annealed to the supplied T7

promoter primer, and filled-in with Klenow fragment to generate a full-length DNA template for transcription into RNA. Two in vitro transcribed RNAs (one the antisense to the other) were generated by in vitro transcription reactions then hybridized to each other to make double-stranded RNA. The double-stranded RNA product was treated with DNase (to remove the DNA transcription templates) and RNase (to polish the ends of the double-stranded RNA), and column purified to provide the three siRNAs that were delivered and tested in cells.

Example 2: Delivery of a small interfering RNA targeting human ataxin1 mRNA.

The constructed siRNA molecules 1-3 described in Example 1 were transfected into HEK293 cells. The RNA produced by the transfected cells was harvested and assayed to measure the amount of human ataxin1 mRNA.

Figure 1 shows the results of a quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) assay for the amount of ataxin1 messenger RNA (mRNA) per microgram of total RNA from cultures of HEK 293H cells. Four cell populations were assayed. The first were 293H cells that had been transiently transfected with siRNA against GAPDH, a "housekeeping gene" with no known relationship to ataxin1 mRNA expression. (The siRNA against GAPDH was supplied as a standard control by Ambion, Inc., in their commercially-available kit for making and testing siRNA). The second were 293H cells that had been transiently transfected with siRNA against ataxin1 mRNA at location 1671 in the ataxin1 mRNA sequence. The third were 293H cells transiently transfected with a plasmid containing a ribozyme against ataxin1 mRNA (which cleaves ataxin1 mRNA at position 1364 in the ataxin1 mRNA sequence). The fourth were 293H cells transiently transfected with siRNA against ataxin1 mRNA at location 0945. All cell populations were harvested concurrently for total cellular RNA, at a time point 48 hours after transfection.

On the gels pictured, the amplified DNA products of the RT-PCR reaction were separated by molecular size, using gel electrophoresis, and are visible as bands of varying intensity. Each cell population described was assayed using a series of parallel reactions, shown as a set of lanes at the top or bottom of each gel. Each set of lanes contains two bands per lane. The top band is the DNA product amplified from a known quantity of

DNA added to the reaction to compete with the endogenous cDNA reverse transcribed from the cellular mRNA. If the bands in a given lane are of the same intensity, then the amount of cellular mRNA in the original cell sample can be inferred to be equivalent to the amount of known quantity of DNA added to the reaction tube. From left to right across the lanes, the amount of known DNA standard added was decreased, in the picogram amounts shown. The assay is interpreted by looking for the set of lanes for which the intensity of the bands "crosses over" from being brightest for the DNA standard, to being brightest for the cellular product below it, indicating that the amount of DNA standard is now lower than the amount of cellular mRNA.

On the gel shown in Figure 1, the top set of lanes is from the cells transfected with the ribozyme against ataxin1 mRNA. The comparison of the bands from this cellular sample to the bands from the DNA standards indicates that the amount of ataxin1 mRNA in these cells is between .505 and .303 picograms per microgram of total cellular RNA. The bottom set of lanes is from the cells transfected with siRNA against ataxin1 at position 0945. Analysis of these lanes indicates that the amount of ataxin1 mRNA in these cells is between .303 and .202 picograms per microgram of total cellular RNA.

On the gel shown in Figure 2, the top set of lanes is from the cells transfected with a control siRNA against GAPDH. Analysis of these lanes indicates that the amount of ataxin1 mRNA in these cells is between .711 and .400 picograms per microgram of total cellular RNA. Finally, the bottom set of lanes is from cells transfected with another siRNA against ataxin1, at position 1671. These lanes indicate that the amount of ataxin1 mRNA in these cells is between 0.404 and 0.303 picograms per microgram of total cellular RNA.

In summary, the results of this particular analysis were:

Treatment	Amount of ataxin1 mRNA (picograms per microgram total cellular RNA)		
	Lower bound	Upper bound	Midpoint Estimate
Control (GAPDH)	0.400	0.711	0.555
Ribozyme (A1364A)	0.303	0.505	0.404
siRNA (AT1671)	0.303	0.404	0.353

siRNA (AT0945)	0.202	0.303	0.252

These data indicate that both the AT1671 and AT0945 siRNA against ataxin1 were effective at reducing the amount of ataxin1 mRNA in these cells within 48 hours after transfection, and that the siRNA were more effective at the reduction of ataxin1 mRNA than was this anti-ataxin1 ribozyme.

It should be noted that the exemplified method for constructing the small interfering RNA to be used as the therapeutic agents in the invention (that is, assembly from oligonucleotides using in vitro transcription and hybridization) is only one possible means for making the therapeutic small interfering RNA. Other larger scale, more efficient methods for manufacturing small interfering RNA may be used to produce the clinical grade and clinical quantities used for treating human patients, without altering the essence of the invention or departing from the spirit and scope of this invention, as set forth in the appended claims.

Example 3: Allele-Specific Reduction of Ataxin1 Expression Using Small, Interfering RNA

In heterozygous patients, if a single nucleotide polymorphism (SNP) were to differ between the mutant and normal length allele, an appropriate siRNA might selectively reduce expression of only the mutant allele. We have tested 293, DAOY, SK-N-SH and HeLa cells using allele-specific RT-PCR for a SNP at position +927 downstream form the SCA1 start codon (see Accession NT_007592). HeLa cells express a 927C but no 927T allele, while 293 cells express a 927T but no 927C allele. DAOY and SK-N-SH cells express both allelic variants. We have created allele-specific siRNA centered at this site. Results of assays for allele-specific suppression of endogenous SCA1 mRNA by these siRNA variants will be presented.

Example 4 : Construction of Small, Interfering RNA Viral Vectors

A selectable reporter plasmid, pAAV-U6-Tracer is constructed for cloning siRNA. (See Figure 3). The plasmid pAAV-U6-Tracer was constructed to contain the inverted terminal repeats (ITR) of adeno-associated virus, flanking the U6 RNA

polymerase III promoter from pSilencer (Ambion), and the EF1a promoter, green fluorescence protein, Zeocin^r resistance, and SV40 poly A from pTracer (Invitrogen). The gene segments are cloned as shown in Figure 3. Oligonucleotides for expressing siRNA are cloned into the multiple cloning region just downstream in the 3' direction from the U6 RNA polymerase III promoter.

HEK293 Cells are cotransfected with pAAV-siRNA, pHelper, and pAAV-RC to make viral producer cells, where the pAAV-RC and pHelper plasmids are part of the three plasmid AAV production system Avigen, Inc.) . The producer 293 cells are grown in culture are used to isolate recombinant viruses, which is used to transfect secondary cells: HeLa Cells, DAOY cells, and SK-N-SH cells.